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BACLOFEN AND GAMMA-HYDROXYBUTYRATE DIFFERENTIALLY ALTERED BEHAVIOR, EEG ACTIVITY AND SLEEP IN RATS

A. HODOR,[†*] S. PALCHYKOVA, B. GAO AND C. L. BASSETTI

Center for Experimental Neurology (ZEN), Department of Neurology, Inselspital, Bern University Hospital, Switzerland

Abstract—*Objectives:* Animal and human studies have shown that sleep may have an impact on functional recovery after brain damage. Baclofen (Bac) and gamma-hydroxybutyrate (GHB) have been shown to induce physiological sleep in humans, however, their effects in rodents are unclear. The aim of this study is to characterize sleep and electroencelphalogram (EEG) after Bac and GHB administration in rats. We hypothesized that both drugs would induce physiological sleep.

***Methods:* Adult male Sprague-Dawley rats were implanted with EEG/electromyogram (EMG) electrodes for sleep recordings. Bac (10 or 20 mg/kg), GHB (150 or 300 mg/kg) or saline were injected 1 h after light and dark onset to evaluate time of day effect of the drugs. Vigilance states and EEG spectra were quantified.**

***Results:* Bac and GHB induced a non-physiological state characterized by atypical behavior and an abnormal EEG pattern. After termination of this state, Bac was found to increase the duration of non-rapid eye movement (NREM) and rapid eye movement (REM) sleep (~90 and 10 min, respectively), reduce sleep fragmentation and affect NREM sleep episode frequency and duration ($p < 0.05$). GHB had no major effect on vigilance states. Bac drastically increased EEG power density in NREM sleep in the frequencies 1.5–6.5 and 9.5–21.5 Hz compared to saline ($p < 0.05$), while GHB enhanced power in the 1–5-Hz frequency band and reduced it in the 7–9-Hz band. Slow-wave activity in NREM sleep was enhanced 1.5–3-fold during the first 1–2 h following termination of the non-physiological state. The magnitude of drug effects was stronger during the dark phase.**

***Conclusion:* While both Bac and GHB induced a non-physiological resting state, only Bac facilitated and consolidated sleep, and promoted EEG delta oscillations thereafter. Hence, Bac can be considered a sleep-promoting drug and its effects on functional recovery after stroke can be evaluated both in humans and rats.**

Key words: Baclofen, GHB, sleep, EEG, rats.

INTRODUCTION

Sleep may promote recovery after brain damage. Our recent studies in animals and humans showed negative effects of sleep deprivation and sleep disturbances on brain recovery after stroke (Gao et al., 2008, 2010; Bassetti and Hermann, 2011; Zunzunegui et al., 2011; Cam et al., 2013). Data on the positive effects of sleep promotion are rare.

Gamma-hydroxybutyrate (GHB), an endogenous metabolite of gamma-aminobutyric acid (GABA), is found in all regions of the mammalian brain and considered to act as a neuromodulator or neurotransmitter (Cash, 1994; Maitre, 1997). High doses of exogenous GHB induce sedation, anesthesia and sleep (Cash, 1994; Carai et al., 2001). Therefore, it has been widely used in the clinical practice as an anesthetic adjuvant (Kleinschmidt et al., 1999) and as treatment for narcolepsy with cataplexy (Scrima et al., 1989; Lammers et al., 1993; Fuller and Hornfeldt, 2003; Poryazova et al., 2011). Although most of the effects of exogenously administered GHB are mediated by $GABA_B$ receptors (Carai et al., 2001, 2008; Kaupmann et al., 2003; Vienne et al., 2010), GHB has its own endogenous receptors (Hechler et al., 1992; Castelli et al., 2000) with distinct distribution in the brain (Maitre, 1997; van Nieuwenhuijzen et al., 2009). Yet, the precise function of endogenous GHB remains unknown.

Baclofen (Bac) is another $GABA_B$ receptor agonist, usually used to treat spasticity (Albright et al., 1991; Paisley et al., 2002; Bensmail et al., 2006). It has higher affinity to $GABA_B$ receptors than GHB (Lingenhoehl et al., 1999; Wu et al., 2004). It has been shown that Bac and GHB induced c-Fos expression in distinct brain regions (van Nieuwenhuijzen et al., 2009). Interestingly, only GHB-activated brain areas involved in the regulation of sleep and reward processes. Behavioral effects of the drugs are different. In contrast to GHB, Bac has reduced abuse potential, euphoric effects and does not lead to physical dependence (Kaupmann et al., 2003; Carter et al., 2009).

Bac and GHB affect sleep in humans and animals. It has been shown that GHB decreases sleep latency and increases deep slow-wave sleep (SWS) in humans (Lapierre et al., 1990; Series et al., 1992; Van Cauter et al., 1997; Vienne et al., 2012) and enhances SWS in

*Corresponding author. Address: Center for Experimental Neurology (ZEN), Department of Neurology, Inselspital, Bern University Hospital, Freiburgstrasse 3, 3010 Bern, Switzerland. Tel: +41-0-31-63-20441.

E-mail address: Aleksandra.Hodor@access.uzh.ch (A. Hodor).

[†] A. Hodor is a PhD student of the Neuroscience Center Zürich (ZNZ), University of Zürich, Switzerland.

Abbreviations: ANOVA, analysis of variance; Bac, baclofen; BL, baseline; EEG, electroencelphalogram; EMG, electromyogram; GHB, gamma-hydroxybutyrate; MRN, median raphe nucleus; NREM, non-rapid eye movement; REM, rapid eye movement; SWA, slow-wave activity; SWS, slow-wave sleep.

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rats (Godschalk et al., 1977; Monti et al., 1979). Interestingly, GHB has also been reported to induce hypersynchronous electroencephalogram (EEG) activity in awake humans (Mamelak et al., 1977; Van Cauter et al., 1997) and animals (Godschalk et al., 1977; Stock et al., 1978; Meerlo et al., 2004). Bac increased non-rapid eye movement (NREM) sleep and promoted EEG delta waves during NREM sleep in humans (Darbari et al., 2005; Huang and Guilleminault, 2009; Vienne et al., 2012) and SWS in rats (Manfridi et al., 2001; Ulloor et al., 2004; Darbari et al., 2005; Datta, 2007; Huang and Guilleminault, 2009). The effects of Bac on rapid eye movement (REM) sleep remain controversial. Infusion of Bac into the pedunculopontine tegmental nucleus suppressed REM sleep in rats (Ulloor et al., 2004; Datta, 2007), whereas unilateral Bac infusion into the nucleus basalis of Meynert had no effect on REM sleep (Manfridi et al., 2001). Recent studies in mice have demonstrated that both drugs induce a sub-anesthetic state different from that of physiological sleep (Meerlo et al., 2004; Vienne et al., 2010). However, definitive conclusions remain difficult to achieve, with some studies suggesting that there are physiological sleep-promoting effects of Bac and GHB in rats, while other studies reach contradicting conclusions.

The aim of the present study was to investigate the effect of Bac and GHB on behavior, vigilance states and EEG pattern in rats, to evaluate their sleep-promoting properties and their possible therapeutic potential. We hypothesized that both drugs would induce sleep in rats. To assess a diurnal influence on the magnitude of the effects, the drugs were injected at the onset of the light and dark phase, which corresponds to periods of rest and activity in the rats. This study may assist in establishing an optimal dose and timing for drug administration, thereby maximizing drug benefits and minimizing side effects.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague-Dawley rats ($n = 26$; Harlan Laboratories, Netherlands), weighing 250–270 g at the beginning of the experiment, were maintained on a 12-h light–dark cycle (light onset at 08:00 or 09:00) and 22 ± 0.5 °C ambient temperature. They were kept individually in Makrolon cages and provided with food and water *ad libitum*. The experiments were carried out with governmental approval according to local guidelines for the care and use of laboratory animals in the University Hospital Zürich, Switzerland (where A.H., S.P., B.G. and C.B. worked, when the experiments were conducted).

Surgery

Under deep isoflurane anesthesia (2% isoflurane in 30% O_2 and 70% N_2O), all rats were implanted epidurally with EEG and electromyogram (EMG) electrodes. Four gold-plated mini-screws (0.9 mm in diameter) were positioned in the skull over the motor cortex of the right and left hemispheres (±2 mm to bregma, 2 mm lateral to midline). Electrodes were connected to stainless steel wires and fixed to the skull with dental cement. Two gold wires (0.2 mm in diameter) were inserted bilaterally in the neck muscles for EMG recording. At least 8–10 days were allotted for recovery from surgery before the experiment.

Drugs

Racemic Bac (Sigma–Aldrich Chemie GmbH, Buchs, Switzerland) and GHB (Xyrem® – Sodium Oxybate, donated by UCB-Pharma) were diluted in saline (0.9% NaCl) to obtain 3 mg/ml Bac and 100 mg/ml GHB stock solutions. Two doses of each drug (10 or 20 mg/kg of Bac and 150 or 300 mg/kg of GHB) were administered intraperitoneally (i.p.) with an injection volume of 3.3 and 6.6 ml/kg of Bac and 1.5 or 3 ml/kg of GHB, respectively. The doses were chosen based on the previous studies showing sedative effects of both substances (Meerlo et al., 2004; Koek et al., 2005; Vienne et al., 2010).

Experimental protocol

Rats were subdivided into five treatment groups, including Bac-10 ($n = 6$), Bac-20 ($n = 3$), GHB-150 ($n = 6$), GHB-300 ($n = 5$), and saline ($n = 6$; Fig. 1). Drugs were injected 1 h after light and dark onset. Each rat received two identical injections (one during the light phase and one during the dark phase) 2–3 days apart to allow drug

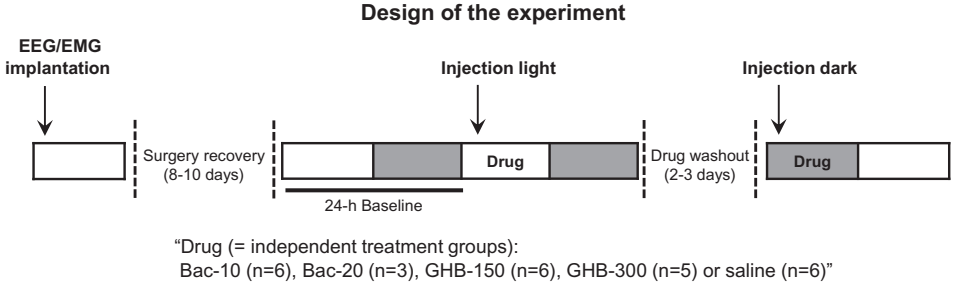

Fig. 1. Design of the experiment. Five treatment groups were designed: baclofen 10 mg/kg (Bac-10, $n = 6$), baclofen 20 mg/kg (Bac-20, $n = 3$), GHB 150 mg/kg (GHB-150, $n = 6$), GHB 300 mg/kg (GHB-300, $n = 5$) and saline ($n = 6$). EEG and EMG were recorded during a 24-h baseline day and following drug injections performed 1 h after light and dark onset. Every rat received two intraperitoneal injections of the drug. At least 2–3 days were allowed for drug washout between injections. 12-h light and 12-h dark phase are indicated by white and gray bars, respectively.

washout (Bac: half-life 3–4 h; GHB: 60 min). After each injection rats were visually observed by the investigator and their behavior was video recorded. Twenty-four hours EEG and EMG recordings were performed during baseline (BL) and after drug administration in each treatment group.

EEG recording and analysis

EEG and EMG were sampled at 200 Hz, signals were amplified, filtered and converted into analog-to-digital signals. Hardware EMBLA and Somnologica-3 software (Medcare Flaga, Iceland) were used. Activity in the 50-Hz band was discarded from the analysis because of power line artifacts. Power spectra of the EEG were obtained by a discrete Fourier transformation (range: 0.75–25 Hz; frequency resolution: 0.25-Hz bins; time resolution: consecutive 4-s epochs; window function: hamming). Three vigilance states – NREM sleep, REM sleep and wakefulness – were scored visually with 4-s epochs. Standard criteria were used to identify vigilance states (Tobler et al., 1997). Briefly, wakefulness was characterized by fast, low voltage desynchronized EEG associated with high-voltage EMG; NREM sleep by high-voltage slow activity in the delta range (0.75–4.0 Hz) associated with low-voltage EMG and REM sleep by low-voltage desynchronized EEG in the theta range (4.0–8.0 Hz) with very low-voltage EMG. Epochs were assigned to a specific vigilance state when more than half of the epoch fulfilled the criteria for that state. Epochs containing EEG artifacts were excluded from spectral analysis in both derivations (14% of recording time, most of them (11%) occurred during wakefulness). In addition to conventional vigilance states we introduced also the drug-induced state, following Bac and GHB administration. This state was characterized by atypical behavior and abnormal hypersynchronous EEG pattern. The first 4-s epoch following drug administration was taken as the onset of the non-physiological vigilance state and the last epoch of abnormal EEG (determined by visual inspection of EEG) was taken as the end of the state. Our analysis was focused on the time period following the end of this state.

Three recording periods were scored and evaluated for every animal: 24-h BL, 11 h following injection during the light phase and 11 h following injection during the dark phase. Investigators scoring the EEG data were blinded to experimental conditions.

Statistical analysis

Drug and time of day effects were evaluated by a repeated-measures or mixed-models analysis of variance (ANOVA) (SAS software, SAS Institute, Cary, NC, USA) followed by paired *t*-test for within subject comparisons (e.g. BL *vs* drug; time of day effect), unpaired *t*-test or Kruskal–Wallis (unequal variance or not normally distributed data) for between subject (group) comparisons (e.g. Bac-10 *vs* saline), Tukey–Kramer or Bonferroni for within and between subject comparisons, if the results of ANOVA reached statistical significance ($p < 0.05$). All reported values are means ± SEM.

RESULTS

Effects of Bac on behavior, vigilance and EEG

Behavior and vigilance states. We first studied the effect of Bac administration on sleep–wake behavior using two different doses (10 mg/kg and 20 mg/kg). Both doses of Bac induced similar behavioral responses, which differed in the magnitude and duration: rats were lying down immobile, their body posture was unnaturally flat with limbs stretched sideways, while the eyes remained open (Fig. 2A). This behavioral response occurred 5–10 min after drug injection and lasted for 40–70 min. Diurnal time of the drug administration had no effect on behavioral response. Concomitant to this behavioral response, we found that Bac induced specific EEG pattern characterized by hypersynchronous slow waves (Fig. 2B), which progressively predominated EEG. The ''drug-induced'' states lasted 246 ± 38 and 216 ± 24 min after Bac-10 (light and dark phase, respectively) and 362 ± 18 and 430 ± 48 min after Bac-20.

When acute effects of the drug on behavior and EEG vanished, normal vigilance states could again be determined. Therefore, long-term effects of the drug on the total amount of wakefulness, NREM sleep, REM sleep and corresponding EEG spectra were evaluated after the termination of the non-physiological ''drug-induced'' state. Bac-10 affected the amount of vigilance states. We found an increase of NREM sleep during the light and dark phase and REM sleep during the dark phase compared to the corresponding BL values ($p < 0.05$, paired *t*-test; Table 1). The drug effect on NREM sleep was stronger during the dark phase (91.4 ± 8.1 vs. 15.7 ± 6.0 min, $t_{(10)} = -7.44$, $p < 0005$, paired *t*-test; values were computed as a difference between treatment and corresponding BL for the light and dark phase). Moreover, Bac-10 administration enhanced significantly the amount of NREM and REM sleep during the dark phase, when compared to the saline treatment ($t_{(10)} = -4.12$, $p < 0.005$ and $t_{(10)} = -1.53$, $p < 0.05$, respectively, unpaired *t*-test; Fig. 3A).

The duration and frequency of NREM sleep episodes were affected by the timing of Bac administration. Thus, during the light phase Bac-10 increased the duration of the episodes ($t_{(10)} = -4.32$, $p < 0.01$, paired *t*-test; Fig. 3B), but reduced their frequency ($t_{(10)} = -2.45$, $p < 0.05$), while during the dark phase it increased the frequency of the episodes ($t_{(10)} = -7.33$, $p < 0.0001$) without changing their duration. Moreover, the number of NREM sleep episodes was significantly different from saline treatment in the dark phase ($t_{(10)} = -4.145$, $p < 0.01$, unpaired *t*-test; Fig. 3C). In addition, Bac-10 administered during the dark phase reduced sleep fragmentation (defined as the occurrence of waking episodes < 16 s per hour of sleep) compared to corresponding BL value (43.1 ± 3.9 vs. 83.9 ± 4.3,

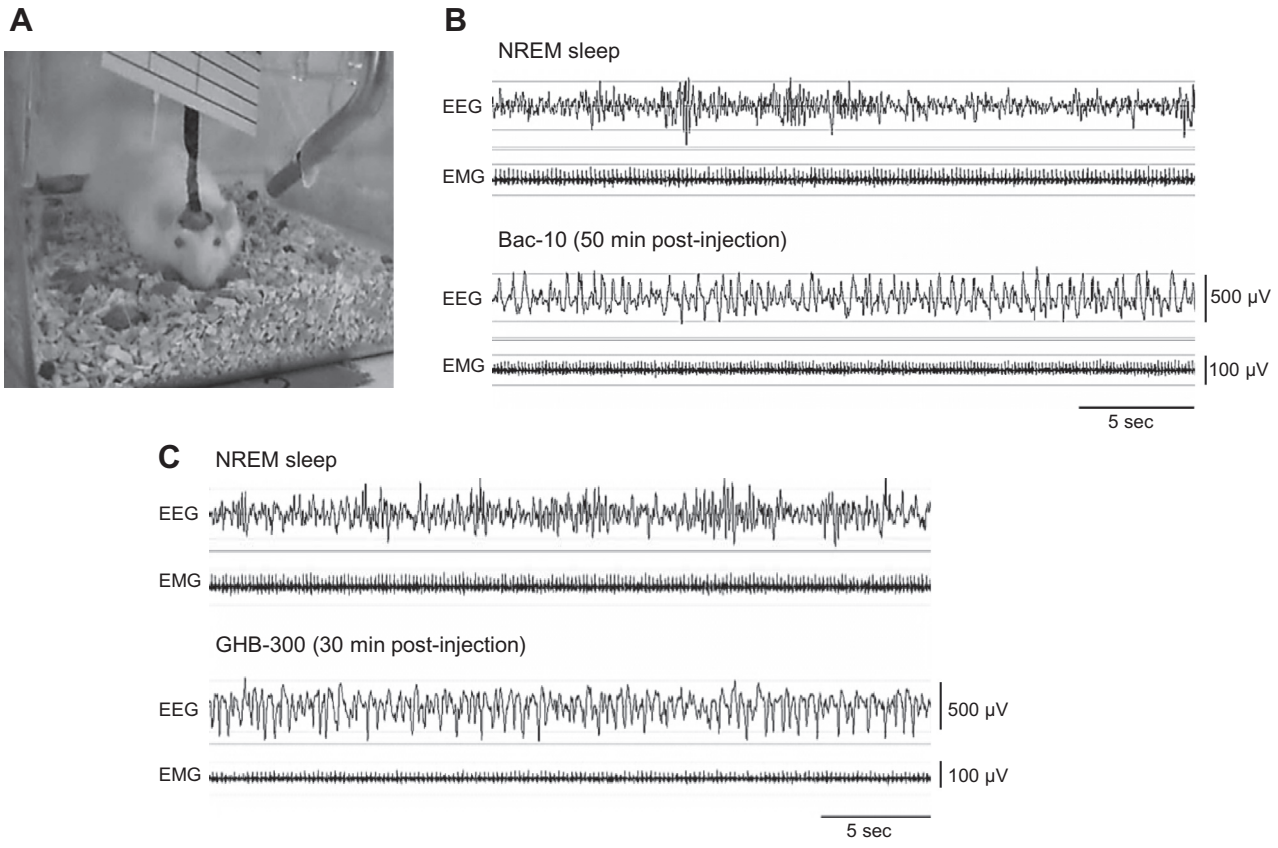

Fig. 2. Effect of baclofen 10 mg/kg (Bac-10) and GHB 300 mg/kg (GHB-300) on behavior and EEG pattern. (A) The picture of the rat 30 min after Bac-10 administration: atypical flat body posture with eyes open. (B) Representative 30-s raw EEG and EMG traces during physiological NREM sleep and following Bac-10 administration in rats. (C) Representative 30-s raw EEG and EMG traces during physiological NREM sleep and following GHB-300 administration in rats.

Table 1. Effects of Bac and GHB on vigilance states

Drug		Wakefulness		NREMs		REMs	
		BL	Treatment	BL	Treatment	BL	Treatment
Bac-10	L	125.14 ± 10.82	110.53 ± 15.27	230.44 ± 23.37	246.1 ± 19.64*	58.34 ± 5.95	57.3 ± 4.45
	D	323.18 ± 7.22	220.08 ± 13.76**	100.86 ± 14.57	192.29 ± 19.19**	19.56 ± 4.41	31.22 ± 3.82*
Bac-20	L	126.13 ± 6.13	103.64 ± 14.05	134.44 ± 5.3	157.07 ± 6.26*	37.89 ± 8.64	37.76 ± 4.65
	D	174.47 ± 16.03	164.47 ± 16.99	61.47 ± 18.92	80.82 ± 13.76	14.24 ± 7.96	4.78 ± 2.3
GHB-150	L	195.83 ± 12.53	173.79 ± 10.18	337.59 ± 12.49	357.34 ± 10.09	77.11 ± 8.69	79.40 ± 7.81
	D	378.11 ± 20.21	361.49 ± 23.02	196.76 ± 17.81	207.34 ± 18.25	34.03 ± 6.33	40.07 ± 5.73
GHB-300	L	195.97 ± 13.42	180.72 ± 11.20	307.40 ± 7.22	313.41 ± 4.33	59.33 ± 5.73	68.51 ± 6.18
	D	359.27 ± 21.03	330.79 ± 20.02	205.97 ± 12.22	233.00 ± 10.35	48.57 ± 9.29	49.99 ± 4.54
Saline	L	236.19 ± 12.33	229.66 ± 13.89	347.90 ± 10.38	346.20 ± 13.25	75.91 ± 4.98	84.14 ± 5.04*
	D	432.84 ± 24.50	422.04 ± 15.93	195.27 ± 21.39	206.54 ± 13.81	31.89 ± 3.93	31.41 ± 3.69

Amount of wakefulness, NREM sleep (NREMs) and REM sleep (REMs; in minutes) following Baclofen (Bac), GHB and saline administration during the light (L) and dark (D) phase. The amount of vigilance states was computed for the period after the end of the non-physiological vigilance states and compared with the corresponding baseline (BL) values. Rats were assigned to the following treatment groups: Bac 10 mg/kg (Bac-10, $n = 6$), Bac 20 mg/kg (Bac-20, $n = 3$), GHB 150 mg/kg (GHB-150, $n = 6$), GHB 300 mg/kg (GHB-300, $n = 5$) and saline ($n = 6$). Asterisks indicate differences between BL and treatment: $^{*}p < 0.05$, $^{**}p < 0.001$, paired t-test.

respectively; $t_{(10)} = -7.69$, $p < 0.001$, paired t-test) or to the saline treatment (40.9 ± 5.3 vs. 16.9 ± 12.1, respectively; $t_{(10)} = 2.37$, $p < 0.01$, unpaired t-test; values represent difference between treatment and corresponding BL). Bac-20 increased NREM sleep during the light phase ($p < 0.05$, paired t-test; Table 1).

EEG spectra and slow-wave activity (SWA) in NREM sleep. Interestingly, we found that Bac-10 administration induced prominent changes in the EEG spectra during NREM sleep. EEG power density in NREM sleep was increased significantly above corresponding time-matched saline values in the 2.25–5.5 and 10.75–14.75-Hz frequency bands during both the light and dark phase ($p < 0.05$, unpaired t-test; Fig. 4A). Moreover, power increase was significantly higher after drug injection in the dark phase compared to the one in the light phase in the frequencies above 3.25 Hz

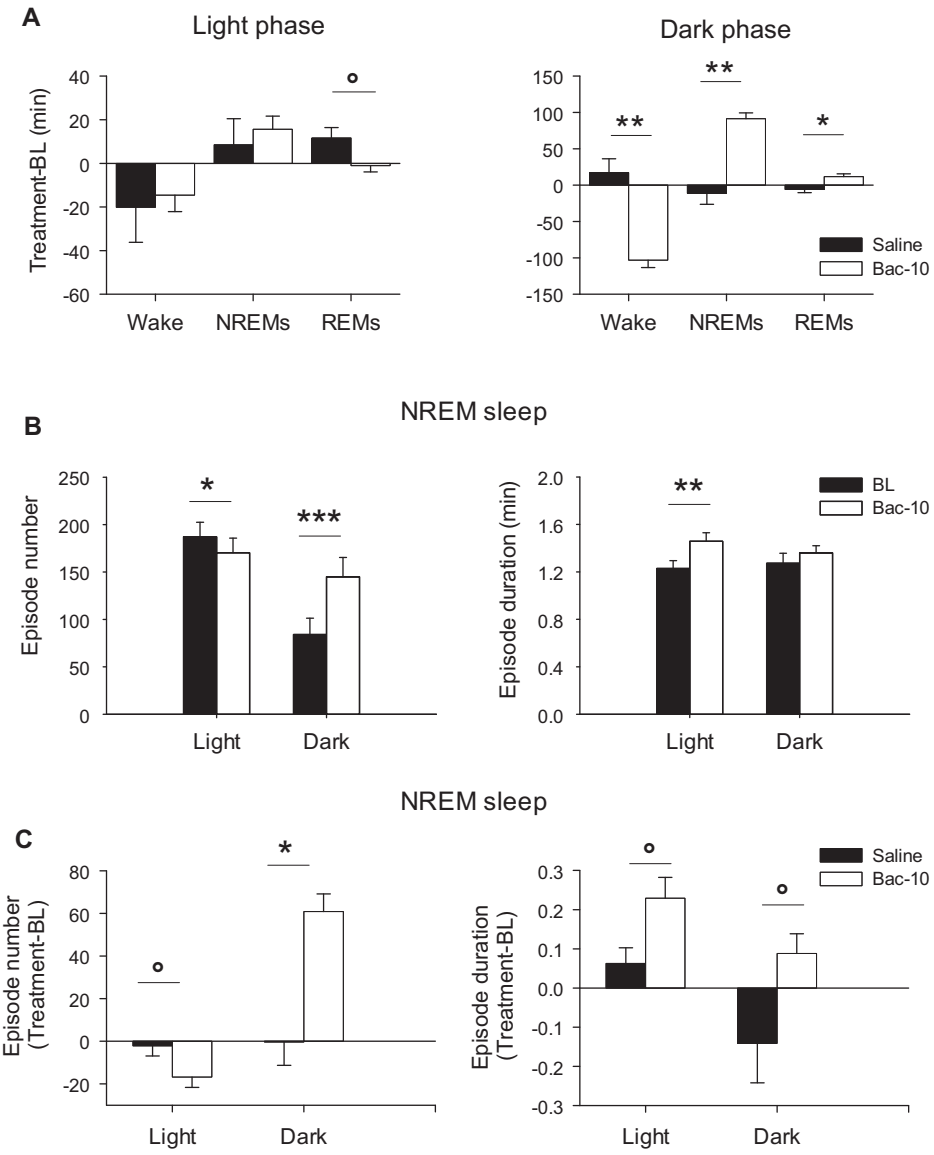

Fig. 3. Effect of baclofen 10 mg/kg (Bac-10) on vigilance states. (A) Amount of wakefulness (Wake), NREM sleep (NREMs) and REM sleep (REMs; in minutes) following Bac-10 ($n = 6$) administration during the light and dark phase compared to the saline treatment ($n = 6$). Mean ± SEM values were computed as a difference between treatment and corresponding baseline (BL) for the period after the end of the non-physiological vigilance states. Bac-10 vs. saline: $^{\circ}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.005$, unpaired *t*-test. (B) Number and duration (in minutes) of NREM sleep episodes following Bac-10 administration during the light and dark phase (baseline comparison). Mean ± SEM values were computed as a difference between treatment and corresponding time-matched BL for the period after the end of the non-physiological vigilance states. Treatment vs. BL: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$, paired *t*-test. (C) Number and duration (in minutes) of NREM sleep episodes following Bac-10 and saline administration during the light and dark phase (saline comparison). Values were computed as a difference between treatment and corresponding time-matched BL for the period after the end of the non-physiological vigilance states. Treatment vs. saline: $^{\circ}p < 0.1$, $^{*}p < 0.01$, unpaired *t*-test.

($p < 0.05$, paired *t*-test; data were normalized to the 12-h BL light phase; not shown). In waking, significant reduction of EEG power was observed after Bac-10 administration compared to saline in the dark phase (1–20.5 Hz; Fig. 4B). No such effect was observed in the light phase. Again, Bac administration induced stronger power reduction during the dark phase compared to the light phase in the frequencies between 1.25–6.75 and 8.25–18.25 Hz ($p < 0.05$, paired *t*-test; not shown). Bac-10 also affected EEG activity during REM sleep (Fig. 4C). It enhanced power density in theta (6.5–8.75 Hz) and beta (above 20.75 Hz) frequencies in the dark phase compared to saline. The light–dark power difference was found in the frequencies 6.75–7.5 Hz ($p < 0.05$, paired *t*-test; not shown). Bac-20 had no significant effect on EEG spectra (not shown).

Further we show that Bac-10 increased sleep intensity based on the analysis of the time course of SWA (0.75–4 Hz) in NREM sleep. SWA was enhanced above the saline level during first 2 h after light or dark Bac-10 injection (Fig. 4D). The Bac-10 effect was stronger during the dark phase ('phase' $p < 0.05$, Tukey–Kramer after mixed ANOVA factor 'phase' $F_{(1,15)} = 5.36$, $p = 0.0351$; not shown).

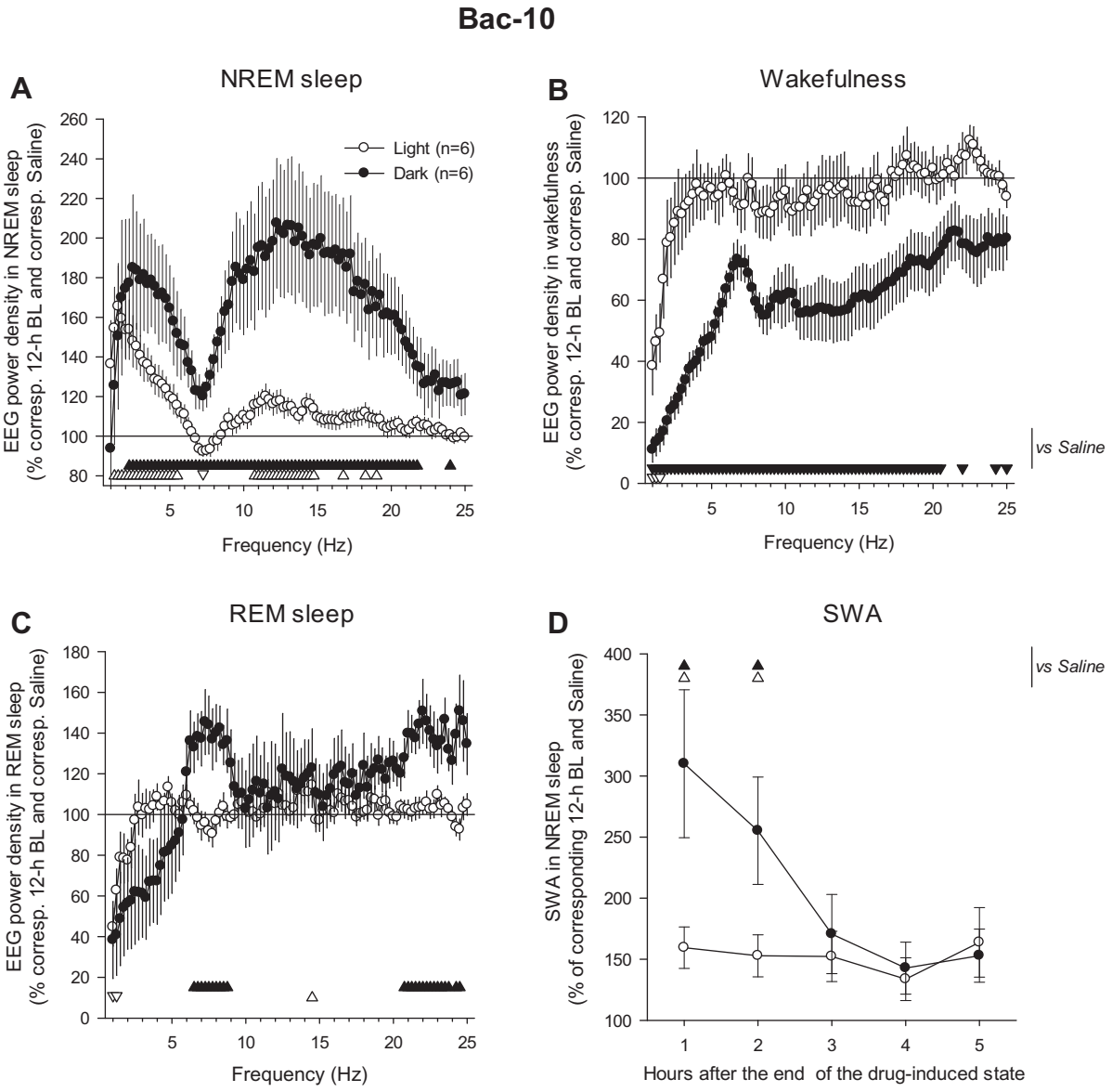

Fig. 4. Effect of baclofen 10 mg/kg (Bac-10) on EEG spectra. EEG power density in NREM sleep (A), wakefulness (B) and REM sleep (C) following Bac-10 ($n = 6$) administration during the light (open circles) and dark phase (filled circles). Power in each frequency bin after Bac-10 or saline treatment ($n = 6$) was first normalized to the corresponding mean 12-h light or dark baseline value of the same bin. Thereafter Bac-10 values were expressed as percentage of power after saline treatment. The curves connect mean values ± SEM computed for the period after the end of the non-physiological vigilance states. (D) Time course of slow–wave activity (SWA) in NREM sleep following Bac-10 administration during the light (open circles) and dark phase (filled circles). Mean ± SEM 1-h values were first expressed as percentage of the corresponding 12-h light or dark baseline SWA in NREM sleep and then as percentage of SWA in NREM sleep after saline treatment. Differences between Bac-10 and saline during the light and dark phase are indicated by white and black triangles, respectively; orientation of the triangles points to the direction of the difference: $p < 0.05$ (unpaired t-test following significant ANOVA).

Effects of GHB on behavior, vigilance and EEG

Behavior and vigilance states. We then studied the effect of GHB administration on sleep–wake behavior using two different doses (150 mg/kg and 300 mg/kg). We found that administration of GHB-300 resulted in a similar behavioral response as both doses of Bac. GHB-150 did not affect animal behavior (rats remained awake, responded to stimuli and moved around the cage). Similar to Bac, GHB-300 induced abnormal, hypersynchronous EEG pattern (Fig. 2C). GHB-150 administration was followed by bursts of hypersynchronous slow waves appearing irregularly. Therefore, our results suggest that GHB induced behavioral state distinct from physiological sleep or wakefulness. The duration of non-physiological states depended on the dose applied. The states lasted 50 ± 10 and 51 ± 5 min after GHB-150 (light and dark phase, respectively) and 97 ± 6 and 46 ± 19 min after GHB-300.

When acute effects of the drugs on behavior and EEG vanished, normal vigilance states could again be

determined. Thus, long-term effects of the drugs on vigilance states and EEG spectra were evaluated after the termination of the non-physiological ''drug-induced'' state. We found that GHB had no major effect on the amount of NREM sleep, REM sleep or wakefulness (Table 1).

EEG spectra and SWA in NREM sleep. We found that GHB application increased EEG power density in NREM sleep in the frequencies encompassing SWA range. However, significance was reached only in the dark phase after GHB-150 (Fig. 5A) and in the light phase after GHB-300 administration (Fig. 5D). Both doses of GHB affected waking EEG power in the dark phase. GHB-150 inhibited power only in the low frequencies (Fig. 5B), while the power in the almost entire frequency range was reduced by GHB-300 (Fig. 5E). GHB had no effect on waking spectra during the light phase. REM sleep EEG spectrum was increased in 7.75–9.5 Hz after GHB-150 administered in the dark phase (Fig. 5C). Minor light–dark differences in the EEG power were observed in the frequencies encompassing 4–4.25 Hz in NREM sleep, 4.5–6.25 and 8.75–13.25 in REM sleep and 5.25–6.5 in waking ($p < 0.05$, paired *t*-test) after GHB-300 injections (not shown).

Recovery from drug effects was also assessed at the level of delta power in NREM sleep. Significant increase of SWA in NREM sleep was observed after GHB-300. SWA was above saline values during first 1-h interval following drug administration in the light phase ($p < 0.05$; Fig. 5G). GHB-150 had no significant effect on sleep intensity (not shown).

DISCUSSION

Our data show that Bac and GHB robustly altered behavior, vigilance states and EEG frequencies in rats. In particular, pharmacological manipulation resulted in a remarkable increase of EEG power in NREM sleep below 5 Hz after the termination of the drug state, pointing to the ability of the drugs to stimulate mechanisms generating EEG NREM sleep oscillations. EEG power in NREM sleep in the higher frequencies was also enhanced by both drugs. Moreover, we show for the first time that the diurnal timing of Bac administration affected the response of rats to these drugs. Specifically, treatment at the beginning of the dark phase induced larger changes in vigilance states and EEG spectra. Our results confirm and extend previous studies investigating the effects of Bac and GHB on changes in sleep and EEG pattern in mice (Meerlo et al., 2004; Vienne et al., 2010).

In animals, like in humans, sleep is homeostatically regulated and the EEG SWA in NREM sleep is used as an indicator of sleep intensity (Borbely, 1982). Our analysis showed that Bac-10, and both doses of GHB, increased NREM sleep intensity after the end of the drug effect. Pharmacological manipulation affected not only EEG, but also led to the increase of sleep duration and consolidation. We cannot exclude that the increase of sleep and its intensity were a consequence of a prolonged drug-induced state, during which sleep pressure continued to accumulate. The increase of SWA following Bac-10 was comparable to the increase observed after a 6-h sleep deprivation (Vyazovskiy et al., 2007). However, drug-induced states were relatively short and lasted on average < 4 h. Moreover, duration of the drug state did not predict the SWA increase (Bac, $n = 12$: $r = 0.11$, n.s.). Therefore, it is unlikely that a ''drug-induced'' state was responsible for the increased sleep intensity observed after the state termination.

NREM sleep EEG changes were not restricted to low frequencies. Thus, Bac-10 enhanced power density in the frequency range encompassing spindles (10.5–21.5 Hz), which might point to increased EEG synchronization within the thalamo-cortical circuit (Terrier and Gottesmann, 1978). GHB-300 reduced power in the frequencies between 6.5 and 8.75 Hz.

It is well known that rodents, in contrast to humans, have a polyphasic sleep–wake pattern. Thus, rats exhibit more sleep during the light phase. Moreover, SWA in NREM sleep is usually high at the beginning of the light phase and reaches its nadir during the dark phase. In this study we applied Bac and GHB at two circadian time points, which are characterized by high and low sleep pressure respectively. Our data indicate that drug administration during low sleep pressure conditions induced stronger changes in sleep and EEG power in NREM sleep. In contrast, treatment at the beginning of the light phase was less effective, probably due to a ceiling effect. Sleep consolidation (i.e. duration and frequency of NREM sleep episodes) was affected differently in the light and dark phase (Fig. 3B, C). The timing of drug administration also influenced the EEG spectra. Light–dark differences were more prominent in waking EEG. Both drugs reduced EEG power in the dark phase (Figs. 4B and 5B, E).

Pharmacokinetics of the drugs might depend on the circadian phase and/or the level of animal activity. Indeed, there are data showing that kinetics can be modified according to the timing of drug administration (Baraldo, 2008). Moreover, a faster metabolism, and therefore, a more transient effect of the drug are expected during the activity phase. That was not the case in our study. In addition, fluctuations in the levels of neurotransmitters might play a role. Hence, it is important to consider the intrinsic sleep need as well as drug kinetics during drug application in experimental and therapeutic conditions.

Consistent with previous studies in mice (Meerlo et al., 2004; Vienne et al., 2010), we showed that both GHB and Bac also induced a drug state distinct from physiological sleep in rats. Several criteria defining normal physiological sleep (Borbely and Tobler, 1989) were affected by the drugs. Specifically, rats consistently displayed unnatural body posture and revealed atypical hypersynchrony in the cortical EEG signal. The magnitude and duration of the observed effects depended on the drug and changed in a dose-dependent manner. The differences in the level of drug response between GHB and Bac are

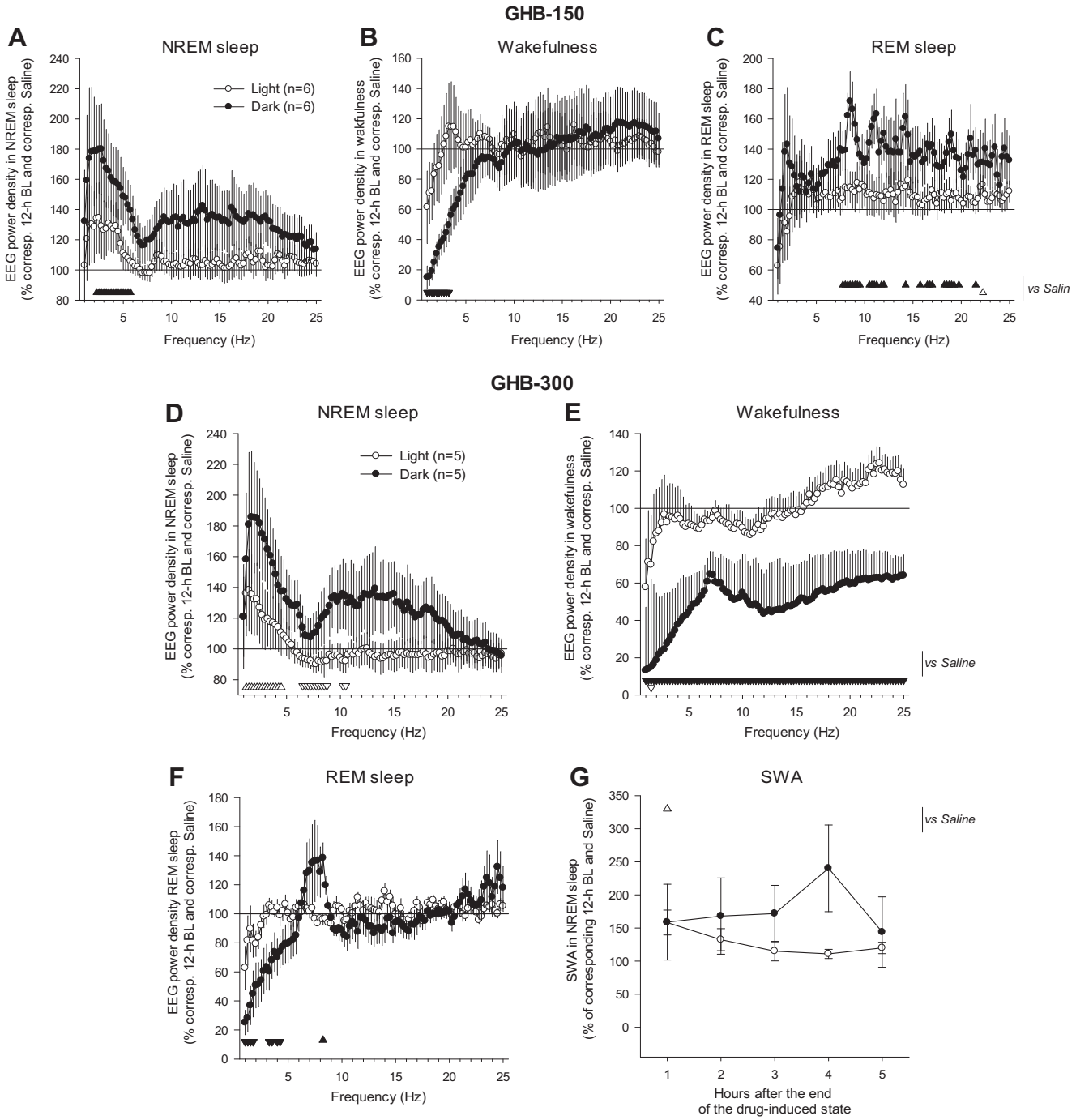

Fig. 5. Effect of GHB 150 mg/kg (GHB-150) and 300 mg/kg (GHB-300) on EEG spectra. (A–C) EEG power density in NREM sleep (A), wakefulness (B) and REM sleep (C) following GHB-150 (n = 6) administration during the light (open circles) and dark phase (filled circles). (D–F): EEG power density in NREM sleep (D), wakefulness (E) and REM sleep (F) following GHB-300 (n = 5) administration during the light (open circles) and dark phase (filled circles). Power in each frequency bin after GHB-150, GHB-300 or saline treatment (n = 6) was first normalized to the corresponding mean 12-h light or dark baseline value of the same bin. Thereafter GHB-150 or GHB-300 values were expressed as percentage of power after saline treatment. The curves connect mean values ± SEM computed for the period after the end of the non-physiological vigilance states. Differences between GHB-150 or GHB-300 and saline treatment during the light and dark phase are indicated by white and dark triangles, respectively; orientation of the triangles points to the direction of the difference: $p < 0.05$ (unpaired t-test following significant ANOVA). (G) Time course of slow-wave activity (SWA) in NREM sleep following GHB-300 administration during the light (open circles) and dark phase (filled circles). Mean ± SEM 1-h values were first expressed as percentage of the corresponding 12-h light or dark baseline SWA in NREM sleep and then as percentage of SWA in NREM sleep after saline treatment. Difference between GHB-300 and saline during the light phase is indicated by white triangle ($p < 0.05$, unpaired t-test following significant ANOVA).

consistent with the half-life of the drugs in rats (60 min for GHB (Snead, 1977; Kueh et al., 2008) and 3–4 h for Bac (Lal et al., 2009)).

In our study GHB treatment had no effect on the amount of vigilance states. This is in line with results shown previously by Meerlo et al. (2004). In contrast, a

recent study in mice reported decreased NREM and REM sleep in the light period following 300 mg/kg of gamma-butyrolactone, a prodrug of GHB (Vienne et al., 2010). Godschalk et al. (1977) showed that doses of 50–100-mg/kg GHB increased SWS, while a dose of 200 mg/kg induced EEG hypersynchrony and behavioral arrest. Studies investigating the 'dose and concentration response relationship' of GHB applied intravenously, reported lack of effect at 150 (Van Sassenbroeck et al., 2001) or 200 mg/kg (Felmlee et al., 2010). Therefore, literature data describing the effects of GHB on sleep is contradictory. The discrepancy might be attributable to experimental conditions, such as the injection time, the amount of data entering the analysis, the route of drug administration, or the species.

Intriguingly, in contrast to GHB, and in agreement with Vienne et al. (2010), Bac increased the amount of NREM sleep in the period following the end of the drug effect. We tested two doses of Bac, i.e. 10 and 20 mg/kg. However, only with the smaller dose was the effect observed. The lack of significance after the higher dose may have various reasons. Firstly, administration of 20 mg/kg of Bac resulted in a long-lasting ''drug-induced'' state, indicating that the rest of the analyzed time after the end of this period was relatively short (3.8–5 h). And secondly, the number of animals we used was small (n = 3), which limited our statistical evaluation.

Our study has limitations. The order of drug administration was fixed. All rats received the first injection during the light phase. However, it is unlikely that this design flaw affected the results due to a short half-life of the drugs (max 4 h) and 2–3-day washout allowed after each injection. The effects of the drugs observed in the present study were so prominent that we could use relatively small sample sizes to show significant effects of the treatment on the most parameters evaluated and, importantly, reduce the amount of animals used in the experiment.

Interestingly, an atypical behavioral state and changes in EEG produced by Bac and GHB-300 have different time courses. Thus, an abnormal EEG pattern appeared a few minutes after each drug administration and lasted up to 97 min for GHB-300 or 7 h for Bac, greatly exceeding the behavioral response (40–70 min for both of the drugs). Drug response might depend on absorption, distribution and elimination rates. After reaching peak plasma concentration, the behavioral effects gradually dissipate, though longer lasting effects in the brain can remain. Therefore, a lower dose of GHB (150 mg/kg) was insufficient to cause any visible behavioral effects, but sufficient to induce changes in EEG.

Both drugs affected EEG power density in REM sleep. A significant increase of power in the theta frequency range was observed after GHB-150 (7.75–9.5 Hz) and Bac-10 (6.5–8.75 Hz) administration in the dark phase (Figs. 5A and 4C). There is evidence that median raphe nucleus (MRN) may serve to desynchronize hippocampal EEG and thus to block theta rhythm (Vertes, 1981). Bac, through $GABA_B$ receptors, might suppress the firing of serotonergic neurons in the raphe nucleus (Colmers and Williams, 1988; Innis et al., 1988) and promote theta rhythms generation. Indeed, Bac infused into the serotonin-containing MRN, promoted theta rhythm in anaesthetized rats (Varga et al., 2002; Li et al., 2005). A number of studies reported increased serotonergic activity, including in the firing rate (Inouye and Kawamura, 1979), c-Fos immunoreactivity (Janusonis and Fite, 2001) and in serotonin release (Kalen et al., 1989; Rueter and Jacobs, 1996) during dark phase. Interestingly, in our study, enhanced theta power was present only during the dark phase.

In addition, GHB and Bac may affect other monoaminergic systems. Thus, both drugs inhibited noradrenergic neurons in locus coeruleus (Osmanovic and Shefner, 1988; Szabo et al., 2004) and dopaminergic neurons in the ventral tegmental area (Madden and Johnson, 1998; Cruz et al., 2004) in rats. It has been suggested that a decrease of dopaminergic or noradrenergic transmission induces an increase in EEG spectral power (Sebban et al., 1999), while stimulation of noradrenergic neurons blocks slow cortical oscillations (Steriade et al., 1993). Moreover, a number of studies reported daily rhythmicity in the activity of those systems, with the peak being in the middle of the dark phase (Nagayama, 1999; Feenstra et al., 2000), which may influence the action of both tested drugs.

CONCLUSION

Our data show that (1) Bac and GHB induce a non-physiological resting state and affect vigilance, EEG and behavior in rats, (2) these effects are dependent on the timing of drug administration, (3) Bac, but not GHB, has sleep-promoting properties. It is possible that observed effects may be related to a complex mechanism engaging multiple neurotransmitter systems. The effects of sleep promotion on functional recovery after a stroke, which is what actually motivated this study, could now be tested using Bac in rats.

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